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Synthesis and Biological Evaluation of Fucoidan-Mimetic Glycopolymers through Cyanoxyl-Mediated Free-Radical Polymerization

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ABSTRACT

The sulfated marine polysaccharide fucoidan has been reported to have health benefits ranging from antiviral and anticancer properties to modulation of high blood pressure. Hence, they could enhance the biological function of materials for biomedical applications. However, the incorporation of fucoidan into biomaterials has been difficult, possibly due to its complex structure and lack of suitable functional groups for covalent anchoring to biomaterials. We have developed an approach for a rapid synthesis of fucoidan-mimetic glycopolymer chains through cyanoxyl-mediated free-radical polymerization, a method suitable for chain-end functionalizing and subsequent linkage to biomaterials. The resulting sulfated and nonsulfated methacrylamido α -L-fucoside glycopolymers' fucoidan-mimetic properties were studied in HSV-1 infection and platelet activation assays. The sulfated glycopolymer showed similar properties to natural fucoidan in inducing platelet activation and inhibiting HSV-1 binding and entry to cells, thus indicating successful syntheses of fucoidan-mimetic glycopolymers.

INTRODUCTION

Fucoidans are one of the most extensively researched classes of marine polysaccharides, as they have been reported to possess pleiotropic functions that include being anti-inflammatory, antiviral, antioxidant, antitumor, anticoagulant, antithrombotic,¹ and an inducer of platelet activation.² This should make fucoidans desirable additives for functionalization of biomaterials for a wide range of biomedical applications. Yet only few reports on fucoidan-infused biomaterials for bone tissue regeneration,³⁻⁵ burn healing,⁶ and antimicrobial studies⁷ exist. The

lack of widespread application in functionalization of biomaterials may be due to the heterogeneous and complex structural features of fucoidans isolated from natural sources.

Fucoidans are isolated from seaweed and marine invertebrates and primarily contain *O*-sulfated α -L-fucosides but also display other structural features such as acetyl groups, proteins and other types of saccharides.⁸ Several recurring motifs have been found in naturally occurring fucoidans (Figure 1). However, these vary from species to species⁸ and also with the season of harvest.⁹ Structure-activity relationships have also been studied regarding sulfation and fucosidic linkage patterns¹⁰⁻¹² as well as molecular weight.⁸ However, firm conclusions have been difficult to draw. For example, Cumashi et al. examined various bioactivities in nine different fucoidans, but were unable to make definite correlations between the activity displayed and the sulfation and fucosidic linkage patterns.¹⁰ Extraction of fucoidan from its natural source often requires harsh conditions thus lowering the accuracy of structural reproducibility.¹³ Also, few chemical groups are found in fucoidans that could serve as anchors for covalent linkage to biomaterials. All these factors raise the need for well-defined and reproducible synthetic fucoidan-mimetics tailored for use in biomaterials.

Figure 1. Fucoidan fragments from *Laminaria saccharina* (a) and *Fucus vesiculosus* (b)¹⁰.

The first synthesis of fucoidan fragments was presented by Jain et al., who synthesized (1→2)-linked difucosides monosulfated in various positions.^{14, 15} Hua et al prepared fully and non-sulfated (1→3)-linked tetrafucosides¹⁶ and a partly sulfated (1→3) and (1→4) alternately linked penta-fucoside.¹⁷ Anticancer studies showed the fully sulfated tetrafucoside to exhibit a higher tumor growth inhibition rate than its nonsulfated counterpart.¹⁶ The penta-fucoside displayed both good antitumor activity *in vivo* and promising anticoagulant activity *in vitro*.¹⁷ The Nifantiev group has presented extensive work synthesizing di-, tri-, tetra-, hexa-, octa-, dodeca- and hexadecafucosides with various differences in branching-, sulfation- and fucosidic linkage patterns.¹⁸⁻²⁵ These fucoidan fragments were subjected to extensive conformational studies through NMR,^{18-23, 25} yet no biological activity studies have been published. We also noted that all the synthetic fucoidan fragments referred to were produced through saccharide formation, which is often time-consuming and results in poor yields with growing saccharide chain length.

As an alternative to saccharide syntheses, glycopolymers are more rapidly synthesized than oligosaccharides and give a higher yield. Glycopolymers are polymers with pendant saccharides formed either through coupling of natural polysaccharides with synthetic polymers,²⁶ polymers formed through initiation from modified polysaccharides,²⁶⁻²⁸ coupling of mono- or oligosaccharides to a polymer chain,²⁹⁻³⁵ or polymers formed from saccharide-pendant polymerizable monomers.³⁰⁻³⁷ Although structurally different from natural polysaccharides, glycopolymers incorporating key structural motifs have been shown to mimic the biological activity of their naturally occurring counterpart.^{34, 35} However, to the best of our knowledge, no reports have been published for the synthesis of fucoidan-mimetics by this approach.

In this paper we present an approach for the formation of long fucoidan-mimetic chains through polymerization of monofucoside-pendant monomers. The fucoidan-mimetic nature of

the resulting polymers was tested through assays for platelet activation and inhibition against Herpes Simplex Virus (HSV-1) infection.

EXPERIMENTAL SECTION

Materials. All chemicals used were purchased from Sigma-Aldrich unless stated otherwise. Merck Silica gel 60 (0.040-0.063 mm) was used for flash column chromatography (FC). Fucoidan from *Fucus Vesiculosus* (crude, M_w 20 000-200 000, product no. F5631) was bought from Sigma-Aldrich and used without further purification. Polyacrylamide (typical M_w 1500, 50 wt % aq.) was bought from Sigma-Aldrich, lyophilized, and used without further purification. Dextran sulfate sodium salt (M_w 500 000) was bought from Pharmacia Fine Chemicals. Heparin sodium salt was bought from Sigma-Aldrich, product no. H3149.

General Methods. Thin-layer chromatography (TLC) was performed on Merck TLC Silica gel 60 F₂₅₄ glass plates and visualized by 254 nm UV light and/or charring after staining with PAA-dip [EtOH (95%, 744 mL), H₂SO₄ (conc., 27.6 mL), AcOH (100%, 8.4 mL), and p-anisaldehyde (20.4 mL)]. ¹H and ¹³C NMR spectra (300 and 75.4 MHz, respectively) were recorded on a Varian 300 spectrometer in CDCl₃ or D₂O at 25°C. The resonances of the deuterated solvent were used as internal standard for all compounds in CDCl₃ (¹H NMR, δ = 7.26; ¹³C NMR, δ = 77.16) and polymers in D₂O (¹H NMR, δ = 4.79); for monomers in D₂O, CH₃OH (¹H NMR, δ = 3.34; ¹³C NMR, δ = 49.50) was used as a reference.³⁸ NMR spectra were processed in MestReNova v8.1.2-11880. Dialysis was performed using Spectra/Por Dialysis Membrane 3 or 6 (MWCO = 3500 and 1000, respectively). Filtering prior to dialysis was done using Acrodisc LC PVDF 0.45 μ m filters. Gel permeation chromatography (GPC) was done at Polymer Standards Service GmbH, Mainz, Germany using a PG16 instrument equipped with a PSS SECcurity 1260 HPLC pump; a PSS PolarSil, 5 μ m, ID 8.0 mm x 50 mm precolumn; three

PSS PolarSil, 5 μm , 100 $\text{\AA}$, ID 8.0 mm x 300 mm columns; and a PSS SECcurity refractive index detector with DMSO / 0.1 M LiBr as eluent at a flow rate of 1.0 mL/min at 70°C. Calibrations were done with dextran/pullulan molar mass standards. Chromatograms were processed using the PSS – WinGPC UniChrom Version 8.1 software. Elemental analysis was performed at Eurofins Mikro Kemi AB, Uppsala, Sweden, for monomers and at Polymer Standards Service GmbH, Mainz, Germany for polymers.

Synthesis of 2-(*N*-*tert*-Butyloxycarbonyl)-aminoethyl 2,3,4-tri-*O*-Acetyl- α -L-Fucopyranoside (2). 2-Azidoethyl 2,3,4-Tri-*O*-acetyl- α -L-fucopyranoside³⁹ (1) (6.58 g, 18.3 mmol), 10% Pd/C (0.301 g) and di-*tert*-butyl dicarbonate (8.02 g, 36.8 mmol) were dissolved in EtOAc (200 mL), stirred at room temperature under H₂(g) atmosphere (1 atm) for 23 h and filtered through Celite. The solid was washed with EtOAc (200 mL) and the combined filtrates evaporated. The crude product was purified through FC (CH₂Cl₂/EtOAc 9:1) which gave *N*-Boc-protected fucoside **2** as a white solid (6.89 g, 15.9 mmol, 87%). *R*_f = 0.46 (CH₂Cl₂/EtOAc 4:1); ¹H NMR (300 MHz, CDCl₃): δ 5.33 (dd, 1H, *J* = 3.5, 10.6 Hz), 5.28 (dd, 1H, *J* = 1.2, 3.5 Hz), 5.12 (dd, 1H, *J* = 3.5, 10.6 Hz), 5.02 (d, 1H, *J* = 3.5 Hz), 4.86 (bs, 1H), 4.14 (q, 1H, *J* = 6.5 Hz), 3.79-3.72 (m, 1H), 3.51-3.44 (m, 1H), 3.41-3.23 (m, 2H), 2.15 (s, 3H), 2.07 (s, 3H), 1.98 (s, 3H), 1.43 (s, 9H), 1.13 (d, 3H, *J* = 6.5 Hz); ¹³C NMR (75.4 MHz, CDCl₃): δ 170.5, 170.2, 169.9, 155.7, 96.4, 79.3, 71.0, 68.1, 67.9, 67.8, 64.5, 40.2, 28.3, 20.6 (two carbons), 20.5, 15.8. Anal. Calcd for C₁₉H₃₁NO₁₀: C, 52.65; H, 7.21; N, 3.23. Found: C, 52.59; H, 7.24; N, 3.14.

Synthesis of 2-Acrylamidoethyl 2,3,4-tri-*O*-Acetyl- α -L-Fucopyranoside (3). To compound **2** (0.157 g, 0.362 mmol) in CH₂Cl₂ (5.0 mL) trifluoroacetic acid (TFA) (1.0 mL) was added and the solution stirred at room temperature for 1.5 h. The solvent was evaporated and residual TFA removed by coevaporation with toluene/MeOH. The resulting syrup was dissolved in CH₂Cl₂

(5.0 mL), and cooled to 0°C whereupon Et₃N (0.202 mL, 1.46 mmol) and acryloyl chloride (0.059 mL, 0.762 mmol) were added. The reaction mixture was allowed to reach room temperature and stirred for 3.5 h. Water was added and the product extracted with CH₂Cl₂. The organic phase was dried over MgSO₄ and the solvent evaporated. FC (CH₂Cl₂/MeOH 49:1) yielded acrylamido fucoside **3** as a slightly yellow syrup (0.097 g, 0.250 mmol, 69%). *R_f* = 0.51 (CH₂Cl₂/MeOH 9:1); ¹H NMR (300 MHz, CDCl₃): δ 6.29 (dd, 1H, *J* = 1.8, 17.0 Hz), 6.10 (dd, 1H, *J* = 10.0, 17.0 Hz), 5.98 (bs, 1H), 5.66 (dd, 1H, *J* = 1.8, 10.0 Hz), 5.35 (dd, 1H, *J* = 3.5, 10.6 Hz), 5.28 (dd, 1H, *J* = 1.2, 3.5 Hz), 5.14 (dd, 1H, *J* = 3.5, 10.6 Hz), 5.05 (d, 1H, *J* = 3.5 Hz), 4.13 (dq, 1H, *J* = 1.2, 6.5 Hz), 3.84-3.75 (m, 1H), 3.63-3.49 (m, 3H), 2.16 (s, 3H), 2.05 (s, 3H), 1.99 (s, 3H), 1.13 (d, 3H, *J* = 6.5 Hz); ¹³C NMR (75.4 MHz, CDCl₃): δ 170.6, 170.3, 170.2, 165.6, 130.7, 126.7, 96.5, 71.1, 68.2, 68.0, 67.3, 64.7, 39.2, 20.8, 20.7, 20.6, 15.9. Anal. Calcd for C₁₇H₂₅NO₉: C, 52.71; H, 6.50; N, 3.62. Found: C, 52.70; H, 6.54; N, 3.51.

Synthesis of 2-Methacrylamidoethyl 2,3,4-tri-*O*-Acetyl- α -L-Fucopyranoside (4). Compound **2** (0.319 g, 0.736 mmol) was dissolved in CH₂Cl₂ (10.0 mL), TFA (2.0 mL) was added and the mixture stirred at room temperature for 1 h. The solvent was evaporated, and residual TFA was removed by coevaporation with toluene/MeOH. The resulting syrup was dissolved in CH₂Cl₂ (20.0 mL) and Et₃N (0.358 mL, 2.58 mmol) and *N*-hydroxysuccinimido (NHS) methacrylate (0.273 g, 1.49 mmol) were added. The reaction mixture was protected from light, stirred at room temperature for 2 h, and evaporated. The product was purified by FC (CH₂Cl₂ → CH₂Cl₂/MeOH 9:1) to give methacrylamido fucoside **4** (0.240 g, 0.598 mmol, 81%) as a colorless syrup. *R_f* = 0.73 (CH₂Cl₂/MeOH 9:1); ¹H NMR (300 MHz, CDCl₃): δ 6.22 (bs, 1H), 5.66 (d, 1H, *J* = 1.2 Hz), 5.33 (dd, 1H, *J* = 3.5, 10.6 Hz), 5.32 (d, 1H, *J* = 1.2 Hz), 5.26 (dd, 1H, *J* = 1.2, 3.5 Hz), 5.12 (dd, 1H, *J* = 3.5, 10.6 Hz), 5.03 (d, 1H, *J* = 3.5 Hz), 4.11 (dq, 1H, *J* =

1.2, 6.5 Hz), 3.81-3.72 (m, 1H), 3.60-3.42 (m, 3H), 2.14 (s, 3H), 2.02 (s, 3H), 1.96 (s, 3H), 1.95 (s, 3H), 1.11 (d, 3H, $J = 6.5$ Hz); ^{13}C NMR (75.4 MHz, CDCl_3): δ 170.7, 170.3, 170.2, 168.4, 140.2, 119.6, 96.6, 71.2, 68.3, 68.0, 67.5, 64.9, 39.3, 20.8 (two carbons), 20.2, 18.7, 16.0. Anal. Calcd for $4\text{C}_{18}\text{H}_{27}\text{NO}_9 \cdot \text{CH}_2\text{Cl}_2$: C, 51.86; H, 6.56; N, 3.31. Found: C, 51.77; H, 6.57; N, 3.28.

General Procedure for Deprotection of Triacetylated (Meth)acrylamido Fucosides (A).

To a tri-*O*-acetylated (meth)acrylamido fucoside (1 equiv) in MeOH (0.03 M), NaOMe (1 equiv) was added. The reaction mixture was protected from light and stirred at room temperature for 0.5-3 h until complete deprotection of the acetyl groups (TLC). The mixture was neutralized with Dowex Marathon C (H^+) and filtered. The beads were washed with MeOH and the combined filtrates evaporated. The resulting syrup was dissolved in H_2O and lyophilized.

Synthesis of 2-Acrylamidoethyl α -L-Fucopyranoside (5). Tri-*O*-acetylated fucoside **3** was deprotected according to general procedure A, which gave fucoside **5** (0.042 g, 0.161 mmol, 99%) as a white solid. $R_f = 0.12$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1); ^1H NMR (300 MHz, D_2O): δ 6.28 (dd, 1H, $J = 10.0, 17.0$ Hz), 6.18 (dd, 1H, $J = 1.8, 17.0$ Hz), 5.76 (dd, 1H, $J = 1.8, 10.0$ Hz), 4.87 (d, 1H, $J = 3.5$ Hz), 3.99 (q, 1H, $J = 6.5$ Hz), 3.85-3.73 (m, 4H), 3.64-3.57 (m, 1H), 3.55-3.42 (m, 2H), 1.17 (d, 3H, $J = 6.5$ Hz); ^{13}C NMR (75.4 MHz, D_2O): δ 169.2, 130.5, 128.0, 98.9, 72.4, 70.2, 68.6, 67.3, 66.9, 39.8, 15.9. Anal. Calcd for $3\text{C}_{11}\text{H}_{19}\text{NO}_6 \cdot 2\text{H}_2\text{O}$: C, 48.34; H, 7.50; N, 5.13. Found: C, 48.25; H, 7.46; N, 4.82.

Synthesis of 2-Methacrylamidoethyl α -L-Fucopyranoside (6). Tri-*O*-acetylated fucoside **4** was deprotected according to general procedure A, to give fucoside **6** (2.01 g, 7.28 mmol, 94%) as a white solid. $R_f = 0.17$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9:1); ^1H NMR (300 MHz, D_2O): δ 5.69 (d, 1H, $J = 1.2$ Hz), 5.46 (d, 1H, $J = 1.2$ Hz), 4.87 (d, 1H, $J = 3.5$ Hz), 3.98 (q, 1H, $J = 6.5$ Hz), 3.84-3.73 (m, 4H), 3.63-3.51 (m, 2H), 3.49-3.41 (m, 1H), 1.92 (s, 3H), 1.18 (d, 3H, $J = 6.5$ Hz); ^{13}C NMR

(75.4 MHz, D₂O): δ 172.4, 139.6, 121.5, 98.9, 72.4, 70.2, 68.6, 67.3, 66.9, 39.9, 18.3, 15.9. Anal. Calcd for 3C₁₂H₂₁NO₆•2H₂O: C, 50.17; H, 7.84; N, 4.88. Found: C, 50.29; H, 7.67; N, 4.82.

General Procedure for Polymerization of (Meth)acrylamido Fucosides (B). *p*-

Chloroaniline (1 equiv) was suspended in water (0.04 M), HBF₄ (50 wt % aq., 1.5 equiv) was added, and the solution was stirred at 0°C under Ar(g) atmosphere until complete dissolution of the solid. NaNO₂ (1.5 equiv) was added, and the solution was stirred at 0°C under Ar(g) atmosphere for 30 min. A solution of (meth)acrylamido fucoside monomer (25 equiv) and NaOCN (5 equiv) in degassed H₂O was added, and the solution was stirred at 55°C under Ar(g) atmosphere for 18 h.

Polymerization of 2-Acrylamidoethyl α -L-Fucopyranoside 5 (7). Acrylamido fucoside monomer **5** in degassed H₂O (0.6 M) was polymerized according to general procedure **B**. The solution was diluted with water, dialyzed against water (MWCO = 1000) for 2 days and lyophilized to give glycopolymer **7** (0.235 g, 58%) as a slightly yellow fluffy solid.

Polymerization of 2-Methacrylamidoethyl α -L-Fucopyranoside 6 (8). Methacrylamido fucoside **6** dissolved in degassed H₂O (0.3 M) was polymerized according to general procedure **B**, diluted with water and dialyzed against water (MWCO = 3500) for 4 days. Lyophilization afforded glycopolymer **8** (0.374 g, 47%) as a slightly yellow fluffy solid.

O-Sulfation of Glycopolymer 8 (9). To nonsulfated glycopolymer **8** (0.185 g) dissolved in DMF (25 mL), sulfur trioxide-pyridine (SO₃•pyr) complex (1.63 g, 5 equiv per hydroxyl group) was added. Stirring at room temperature led to instantaneous formation of a precipitate, which was broken up with a spatula. The reaction mixture was sonicated and subsequently stirred at room temperature for 2 days. Cooling in a freezer for 3 h allowed for further precipitation whereupon the solvent was decanted off. The precipitate was dissolved in a solution of NaHCO₃

(2.27 g, 40 equiv per hydroxyl group) in H₂O (20 mL) and stirred at room temperature for 24 h. Excess NaHCO₃ was eliminated by careful additions of Dowex Marathon C (H⁺), the solution filtered, and the beads rinsed with H₂O. The combined filtrates were washed with CH₂Cl₂, concentrated, and filtered. Purification through dialysis (MWCO = 3500) for 4 days, followed by lyophilization afforded partially *O*-sulfated glycopolymer **9** (0.304 g) as a white fluffy solid.

WST-1 Assay. To ensure that the fucoidan-mimetics were noncytotoxic, we examined their effects on cell viability using a colorimetric WST-1 assay (Roche, 11-644-807-001) according to the manufacturer's instructions. The cells were incubated with the cell proliferation reagent WST-1 at 37°C for about 30 min to allow for color development, which was then quantified using a microplate reader at 450 nm (Molecular Devices) (N=3 samples/group). A general linear model was used to make multiple comparisons. Two groups were analyzed: proliferation at 24h versus treatment and concentration of test compound; and proliferation at 48h versus treatment and concentration of test compound. Statistical significance was set at $P \leq 0.05$.

Virus Infection and Titration. HCECs, a human corneal epithelial cell line,⁴⁰ were cultured in keratinocyte-serum free medium (KSFM) containing L-glutamine, human epidermal growth factor (EGF), and bovine pituitary extract (BPE) (Life Technologies, 17005-075). HSV-1 strain F⁴¹ kindly provided from Dr. Earl Brown (University of Ottawa, Canada) was used in this study. Virus propagation was carried out in Vero cells (ATCC, CCL-81) in Dulbecco's modified Eagles's medium (DMEM) medium containing 10% FBS.

To infect HCEC with HSV-1, monolayers of cells in 24-well plates were adsorbed with virus (multiplicity of infection [MOI] = 1 or 0.1) for 1 h at 37°C. After adsorption, unbound virus was removed by gentle washing with phosphate buffered saline (PBS, 0.01 M, pH=7.4), followed by the addition of fresh medium and further incubation at 37°C.

Immunofluorescence Assay (IFA). Cells were fixed with 4% paraformaldehyde for 30 min at 4°C and then stained with primary antibodies at room temperature for 1 h. The anti-HSV-1/2 (Thermo Scientific, PA1-7488) antibody was used in these assays. After being washed with PBS (0.1% Tween-20), the cells were reacted with Alexa Fluor 488-conjugated goat antirabbit secondary antibody. Cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) to stain for nuclei and examined with a Zeiss fluorescent microscope (Zeiss Axio Observer A1).

Measurement of Platelet Aggregation. Heparinized human blood was obtained from Linköping University Hospital's blood bank. The blood was mixed (1:5 volumetric) with an acid-citrate-dextrose solution composed of 71 mM citric acid, 85 mM sodium citrate, 111 mM glucose (pH 4.5) and centrifuged at 150 g for 20 min. The upper platelet rich plasma phase was collected and treated with 100 µM aspirin for 30 min followed by centrifugation at 520 g for 20 min. The pellet of platelets was carefully washed three times with Krebs-Ringer glucose (KRG) buffer (120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, 10 mM glucose (pH 7.3)) and finally resuspended in KRG supplemented with 0.05 U/mL apyrase. Platelet density was adjusted to 2.5×10^8 /mL and extracellular [Ca²⁺] was set to 1 mM immediately before each experiment. All isolation steps were carried out in room temperature.

Measurements of aggregation were performed in aliquots of platelet suspensions (300 µl) at 37°C under stirring condition using a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-log, Haverston, PA, USA). The aggregation responses are expressed as percentage increase in light transmission compared to platelet-free KRG (= 100%).

Scheme 1. Reagents and conditions: (a) Pd/C, H₂, Boc₂O, EtOAc, rt, 23 h, 87%; (b) (i) TFA, CH₂Cl₂, rt, 1.5 h; (ii) acryloyl chloride, Et₃N, CH₂Cl₂, 0°C → rt, 3.5 h, 69% (over two steps); (c) (i) TFA, CH₂Cl₂, rt, 1 h; (ii) NHS-methacrylate, Et₃N, CH₂Cl₂, rt, 2 h, 81% (over two steps); (d) NaOMe, MeOH, rt, 0.5-3 h, 99% (R=H), 94% (R=Me).

RESULTS AND DISCUSSION

Synthesis of (Meth)acrylamido Fucoside Monomers. Striving for simplicity and a rapid synthesis in this first study, our choice of fucoidan-mimetic polymer side chain was an *O*-sulfated mono- α -L-fucoside. Preservation of the native α -fucosidic bond is an important factor in reproducing the biological activity.⁴² Hence, 2-Azidoethyl 2,3,4-Tri-*O*-acetyl- α -L-fucopyranoside (**1**) (Scheme 1) was deemed as a suitable starting material due to its inherent α -fucosidic bond and the accessible terminal azide functionality. **1** was synthesized according to a known procedure³⁹ and the azide reduced to amine by catalytic hydrogenation. Initial reactions gave low yields due to *O*→*N* acetyl group migration and dimerization at the amine, all observed by ¹H-NMR. The dimer was difficult to separate from the desired product and change of catalyst and solvent gave the same results. Employing the method of *in situ* protecting the free amine by addition of Boc₂O⁴³ gave *N*-Boc-protected fucoside **2** in 87% yield without any noticeable

byproducts formed. As functional groups to be polymerized acrylamide derivatives were chosen. Deprotection of the *N*-Boc-group in TFA directly followed by coupling of the free amine with acryloyl chloride produced acrylamido fucoside **3** in 69% yield. This reaction produced several byproducts lowering the yield and complicating the cleanup. As an alternative the free amine was, upon deprotection of the *N*-Boc-group in TFA, coupled with NHS-methacrylate to form methacrylamido fucoside **4**. This procedure gave a more product-specific reaction and increased the yield to 81%. Since both acrylamido and methacrylamido functionalities are susceptible to polymerization both set of fucosides were used in the subsequent steps. Tri-*O*-acetylated fucosides **3** and **4** were then subjected to basic ester hydrolysis in NaOMe/MeOH which gave deprotected fucoside monomers **5** and **6**, respectively. These compounds were observed to self-polymerize when evaporating the MeOH under heating. When protected from heat and light during work-up and storage, no such observations were made. Attempts were made to *O*-sulfate the free hydroxyl groups of the fucoside monomers, but the relatively small size of the tri-*O*-sulfated products prohibited purification by either dialysis or size exclusion chromatography. *O*-sulfation was hence carried out postpolymerization.

Polymerization of (Meth)acrylamido Fucoside Monomers. Several methods for synthesizing chain-end functionalized glycopolymers for covalent binding to biomaterials have been reported.⁴⁴ We selected cyanoxyl-mediated free-radical polymerization due to its mild reaction conditions and tolerance to hydroxyl groups.⁴⁵ This method has demonstrated successful incorporation of various functional groups at the initiating end, and yields an inherent cyanate group at the terminating end, of the glycopolymer chain.⁴⁶ Fucoside monomers **5** and **6** were polymerized with *p*-chloroaniline chosen as the initiating phenylamine due to its demonstrated high yield⁴⁶ and its unreactive chloride group (Scheme 2). The aryl-

type active radical was produced *in situ* in aqueous solution via reaction between cyanate and the aryl-diazonium salt formed by reacting *p*-chloroaniline with fluoroboric acid and nitrite according to the mechanism described by Grande et al.⁴⁷ Subsequent addition of fucoside monomers **5** and **6** yielded glycopolymers **7** (58%) and **8** (47%), respectively, upon purification by dialysis against deionized water. The slightly lower yield of glycopolymer **8** compared to glycopolymer **7** could be attributed to the well-known slower reactivity of methacrylamides than of acrylamides in polymerizations. ¹H-NMR confirmed the polymerization of fucoside monomer **6** by the move of the methacrylate alkene proton signals from 5.69 and 5.46 ppm upfield to form a singlet peak at 1.69 ppm (containing 2 protons) and the move of the methacrylate methyl proton signal from 1.92 ppm upfield to 0.76 ppm (Figure 2). Signals corresponding to the chain-end aryl protons can be found at 7.26 and 7.01 ppm confirming a successful cyanoxyl-mediated free-radical polymerization. In a similar fashion, the move of the acrylate alkene proton signals of fucoside monomer **5** from 5.76, 6.18, and 6.28 ppm upfield to form two singlets at 1.51 (containing 2 protons) and 1.98 ppm (containing 1 proton) confirmed the successful formation of glycopolymer **7**. The GPC elugram for glycopolymer **8** showed one peak (Figure 3), and molecular weights were found to be $M_n=30\,200$ Da and $M_w=65\,100$ Da giving a dispersity of $D=2.15$. The elugram for glycopolymer **7** however showed two peaks. Given these results, glycopolymer **8** alone was furnished to a fucoidan-mimetic glycopolymer by *O*-sulfation.

Scheme 2. Reagents and conditions: (a) *p*-chloroaniline, HBF₄, NaNO₂, NaOCN, H₂O, 0°C → 55°C, 18 h; (b) (i) SO₃•Pyr, DMF, rt, 2 days; (ii) NaHCO₃, H₂O, rt, 24 h.

Figure 2. ¹H-NMR spectrum of glycopolymer **8** in D₂O, 300 MHz.

Figure 3. GPC elugram of glycopolymer **8**.

***O*-Sulfation of Glycopolymer **8**.** To complete the fucoidan-mimetics, glycopolymer **8** was *O*-sulfated through treatment with sulfur trioxide pyridine complex in DMF (Scheme 2). The pyridinium salt of glycopolymer **9** formed almost instantly, precipitated out of solution and was subsequently crushed, sonicated, and further stirred to maximize the *O*-sulfation. The sodium salt product was then yielded through ion-exchange by dissolving the precipitates in a sodium bicarbonate water solution and residual compounds removed by extraction with DCM and dialysis against deionized water. The ^1H -NMR spectrum of compound **9** confirmed the *O*-sulfation by the move of the signals for fucoside protons H-2, H-3 and H-4 (h, i, and j in Figure 2) from 3.75 ppm downfield to 4.50, 4.64 (merged with H_2O), and 4.89 ppm. A downfield move in signal for a proton neighboring a hydroxyl group turned to ester, e.g., a sulfate ester, is well-known, and hence expected and in line with previous reports on *O*-sulfation of α -L-fucopyranosides.^{16, 24} The integral ratio between fucoside, ethylene linker or polymer backbone peaks on ^1H -NMR did not change compared to glycopolymer **8**, indicating that the *O*-sulfation conditions did not hydrolyze the glycosidic nor the amide bonds. Elemental analysis for glycopolymer **9** showed carbon and sulfur contents of 23.01 and 10.18 %, respectively, corresponding to a sulfation degree of 66 %. It should be noted that high water content due to the

hygroscopic sulfate esters might give some discrepancies to these results. To determine their fucoidan-mimetic properties, glycopolymers **8** and **9** were tested in assays for biocompatibility, HSV-1 infection and platelet aggregation.

Biocompatibility of Fucoidan-Mimetic Glycopolymers. Prior to examining the glycopolymers for their biological activities, we first determined the biocompatibility of these compounds with HCECs, as compared to the natural fucoidan, dextran sulfate, heparin and the control polyacrylamide backbone. An important function of corneal epithelial cells is to be able to proliferate and differentiate to replenish the epithelium. We showed that cell proliferation as determined by a WST-1 assay was significant. As shown in Figure 4, cell proliferation was only inhibited by the commercially available natural fucoidan. Effects of a dose of 100 $\mu\text{g/mL}$ were significant ($P \leq 0.05$ by at 24 h after treatment and 48 h treatment).

Figure 4. Effect of glycopolymers **8**, and **9** compared to natural fucoidan, heparin, dextran sulfate and polyacrylamide on cell proliferation as an indication of biocompatibility of these compounds. HCECs were cultured in the presence of 0, 1, 10, and 100 µg/mL of each compound for 24 and 48 h. Cell proliferation as determined by the WST-1 assay showed that the various compounds had no effect on proliferation except for natural fucoidan at 10 and 100 µg/mL levels.

Synthetic Glycopolymers Interfere with HSV-1 Infection. Natural fucoidan has been reported to inhibit coated viruses such as HSV by blocking receptor-mediated entry and interfering with replication.⁴⁸ To determine whether these antiviral activities are retained in our synthetic glycopolymers, we tested the drug effect of glycopolymers in HSV-1-infected HCECs. As a reference, natural fucoidan from *Fucus Vesiculosus* was used and the results compared. Polyacrylamide, heparin, and dextran sulfate were also tested in these assays, the former to exclude any effects from the polymer backbone and the latter for comparison to their documented biological properties. Several sulfated polysaccharides, including heparin and dextran sulfate with documented effects against HSV-1 infection^{12, 49-51} were also tested as benchmarks. We found that when HCECs were cultured in the presence of glycopolymer **9**, natural fucoidan, heparin, and dextran sulfate, they were not infected by HSV-1 viruses that were added to the culture (Figure 5). However, nonsulfated glycopolymer **8** and polyacrylamide did not have any antiviral activity (Figure 5).

Figure 5. Effect of treatment of HCECs with glycopolymer **8**, and **9** (100 µg/mL) compared to natural fucoidan, heparin, dextran sulfate and polyacrylamide. At 24 h post infection, HCECs pretreated with sulfated glycopolymer **9** and other sulfated compounds (heparin, dextran sulfate, and natural fucoidan) showed a marked decrease in the expression of active virus (green) compared to nonsulfated glycopolymer **8**, polyacrylamide, and untreated samples. HCEC cell nuclei were stained with DAPI (blue).

Synthetic Glycopolymers Block HSV-1 Activity during the Virus-Cell Adsorption Step.

We then determined the potential mechanism by which glycopolymer **9** was blocking the herpes infection. HCECs infected with HSV-1 were treated with 100 µg/mL of sulfated glycopolymer **9** at different time points of infection. Pretreatment of cells with sulfated glycopolymers, after which they were washed off had no noticeable antiviral effect (Figure 6A), indicating that the sulfated glycopolymers did not cause any changes in the cell membrane to block viral entry.

However, the HSV-1 viral protein expression was greatly reduced by glycopolymer **9** as well as heparin, dextran sulfate and natural fucoidan when it was added during viral adsorption (Figure 6B). This strongly suggests that both glycopolymer **9**, fucoidan, and the other sulfated compounds reacted with the viral particles and blocked their entry into the cells.

However, post-treatment of already infected cells with glycopolymer **9** or other sulfated compounds had no noticeable antiviral effect (Figure 6C) despite previous claims that natural fucoidan interfered with viral replication.⁵² Furthermore, when we infected HCECs with a low MOI of 0.1 to examine the effect of the glycopolymer **9** on viral spreading, we showed that the sulfated glycopolymer (as well as heparin, dextran sulfate, and natural fucoidan) was able to slow down but not stop the viral spreading (Figure 7). A low MOI results in infection of an

initially small number of cells allowing for observation of viral spreading. Without treatment, most of the HCECs were infected 48 h post infection (Figure 7). With glycopolymer **9** treatment, however, a large number of cells remained unstained by the anti-HSV-1/2 antibody. Collectively, our results suggest that the sulfated glycopolymers most likely influenced the binding and entry of HSV-1 in cells, and therefore slowed down the viral spreading in HCECs.

A. Cell Pretreatment

B. During Adsorption

C. Post Adsorption

Figure 6. Effect of sulfated compounds (100 $\mu\text{g/mL}$) - heparin (a), dextran sulfate (b), natural fucoidan (c), or glycopolymer **9** (d) on HSV-1 infection of cornea epithelial cells (HCECs) under different conditions, at 24 h postinfection. (A) HCECs were pretreated with compounds for 30 min, washed, then infected with HSV-1 (MOI=1). (B) Compounds were added in the culture

medium during viral adsorption. (C) Compounds were added after viral adsorption. Cells infected with HSV-1 are stained green by an anti-HSV-1/2 antibody that binds viral particles. HCEC nuclei were stained with DAPI (blue). Nontreated HCECs (e) are displayed as references.

A. 24 hours

B. 48 hours

C. 72 hours

Figure 7. Glycopolymer **9** inhibited HSV-1 spreading in already infected HCECs. HCECs were infected with HSV-1 at a MOI = 0.1. After 1 h adsorption, the inoculated virus was washed away, and the cells were cultured in fresh medium containing 100 µg/mL of synthetic glycopolymers. At 24, 48, and 72 h post infection, the cells were stained with an anti-HSV-1/2 antibody for viral protein expression (green). Cell nuclei were stained with DAPI (blue). (a) Heparin, (b) dextran sulfate, (c) natural fucoidan, (d) glycopolymer **9**, and (e) nontreated HCEC.

Sulfated Glycopolymers Induce Platelet Activation. The influence of nonsulfated and sulfated glycopolymers was further assessed on isolated human blood platelets using the same reference substances as in the HSV-1 infection studies. Addition of 100 µg/mL of glycopolymer **9** induced an immediate aggregation response of platelets (Figure 8A). The effect of the sulfated glycopolymer was detected down to a dose of 0.3 µg/mL (not shown), and maximal aggregation response was equivalent to that induced by natural fucoidan (Figure 8B). Nonsulfated glycopolymer **8** did not stimulate platelet aggregation (Figures 8A and B), and it is notable that platelets responded normally to subsequent activation by the thrombin receptor-activating hexapeptide SFLLRN (10 µg/mL). Furthermore, heparin and dextran sulfate as well as backbone molecule polyacrylamide had no detectable impact on human platelets (Figure 8B). Taken together, these results show that sulfated but not nonsulfated glycopolymers activated human blood platelets and consequently enhanced primary hemostasis.

Figure 8. Effect of glycopolymers on isolated human platelets. Aliquots of platelet suspensions were exposed to nonsulfated glycopolymer **8** (100 $\mu\text{g/mL}$; green traces) and thereafter activated by the thrombin receptor hexapeptide agonist SFLLRN (10 $\mu\text{g/mL}$). SFLLRN, but not the nonsulfated glycopolymer, induced a prompt increase in light transmission which corresponds to aggregation of platelets. Addition of sulfated glycopolymer **9** (100 $\mu\text{g/mL}$; red traces) caused an immediate and pronounced aggregation response. The maximal increase in light transmission through platelet suspensions following the addition of 100 $\mu\text{g/mL}$ of either glycopolymers **8**, **9**, fucoidan, dextran sulfate, heparin or polyacrylamide are summarized in (B). Platelet aggregation

by SFLLRN (10 $\mu\text{g/mL}$) is shown as a positive control. Data are presented as mean $\pm$ SEM (n=3-4).

Fucoidan-Mimetic Activity Depends on Both Fucoside Moiety and Sulfation. These studies have shown that sulfated glycopolymer **9** has the ability to induce platelet activation and to prevent the binding and entry of HSV-1 to cells, in analogy with naturally derived fucoidan from *Fucus vesiculosus*. Polyacrylamide, which was used as a reference to the polymeric backbone, and nonsulfated glycopolymer **8** showed neither of these biological properties. Although other sulfated oligosaccharides such as dextran sulfate and heparin showed similar abilities to prevent HSV-1 infection, only fucoidan and sulfated glycopolymer **9** possessed the ability to induce platelet aggregation. This confirms that sulfated glycopolymer **9** behaves as a functional mimic of natural fucoidan.

CONCLUSIONS

Methacrylamido α -L-fucoside monomers were successfully synthesized, polymerized, and subsequently partially *O*-sulfated to yield fucoidan-mimetic sulfated and nonsulfated glycopolymers. The fucoidan-mimetic nature of the glycopolymers was established by HSV-1 infection and platelet activation studies.

We conclude that a sulfated fucoside polymer side chain is the key structural element for yielding fucoidan-mimetic properties. We believe this to be a first step toward incorporating the biological properties of fucoidan into biomaterials.

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Notes

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